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Hybridization versus conservation: are domestic cats threatening the genetic integrity of wildcats (*Felis silvestris silvestris*) in Iberian Peninsula?

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Cross-breeding between wild and free-ranging domestic species is one of the main conservation problems for some threatened species. The situation of wildcats (*Felis silvestris silvestris*) in Europe is a good example of this critical phenomenon. Extensive hybridization was described in Hungary and Scotland, contrasting with occasional interbreeding in Italy and Germany. First analyses in Portugal revealed a clear genetic differentiation between wild and domestic cats; however, four hybrids were detected. Here, we extended the approach to Iberian Peninsula using multivariate and Bayesian analyses of multilocus genotypes for 44 Portuguese wildcats, 31 Spanish wildcats and 109 domestic cats. Globally, wild and domestic cats were significantly differentiated ($F_{ST}=0.20$, $p<0.001$) and clustered into two discrete groups. Diverse clustering methods and assignment criteria identified an additional hybrid in Portugal, performing a total of five admixed individuals. The power of admixture analyses was assessed by simulating hybrid genotypes, which revealed that used microsatellites were able to detect 100, 91 and 85% of first-generation hybrids, second-generation genotypes and backcrosses, respectively. These findings suggest that the true proportion of admixture can be higher than the value estimated in this study and that the improvement of genetic tools for hybrids detection is crucial for wildcat conservation.

Keywords: *Felis silvestris*; hybridization; conservation genetics; microsatellites; Bayesian admixture analysis

1. INTRODUCTION

The anthropogenically mediated dispersion of free-ranging domestic cats (*Felis silvestris catus*), and their contact with natural populations of European wildcats (*Felis silvestris silvestris*), is considered one of the main threats for the survival of wildcat populations throughout all Europe. The unknown effects of long-term sympatry between the two subspecies resulted in a global concern regarding the genetic and taxonomic status of the European wildcat (McOrist & Kitchener 1994; Daniels *et al.* 1998; Beaumont *et al.* 2001). The main problems that lead to artificial hybridization are related to habitat fragmentation and home-range changes, scarce availability of prey and the increasing structuring of small and isolated natural populations (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). These factors may have been promoting a more frequent and large-scale contact between wild and domestic cats and a continuous backcrossing of hybrid individuals to parental populations may eventually culminate in a deep and irreversible genetic pollution of wild populations. For example, the swamping of domestic alleles into the wildcat genome over successive generations resulted in high admixture levels in Hungarian and Scottish populations (Beaumont *et al.* 2001; Daniels *et al.* 2001; Pierpaoli *et al.* 2003; Lecis *et al.* 2006). Interestingly, only occasional interbreeding was found in Italy, Germany and Portugal, with wildcat populations clearly differentiated from domestic cats and still preserving their genetic singularity (Randi *et al.* 2001; Pierpaoli *et al.* 2003; Lecis *et al.* 2006; Oliveira *et al.* 2007).

Preventing introgression in wild populations strongly depends on the efficient detection of admixed individuals. Several genetic approaches have been extensively and successfully used to address this problem in different *taxa* (Rhymer & Simberloff 1996; Allendorf *et al.* 2001), especially in cases where phenotypical classifications of hybrid classes or even parental groups are dubious, as occur between wild and domestic cats and their hybrids (Daniels *et al.* 1998; Beaumont *et al.* 2001). The ability to genetically distinguish admixed individuals within sympatric populations of closely related (sub)species can provide invaluable resources for wildlife management, and has proved to be essential in studies of population structure

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One contribution of 16 to a Theme Issue 'Hybridization in animals: extent, processes and evolutionary impact'.

and admixture in wildcat populations (Daniels *et al.* 1998; Oliveira *et al.* 2007). Similarly to most organisms, intraspecific distinction among *Felis silvestris* is barely based on diagnostic genetic differences and, consequently, the identification of parental and hybrid individuals is based on probabilistic assessments (Nielsen *et al.* 2006). The most promising mechanism to study artificial hybridization is the combination of highly informative molecular markers with model-based Bayesian software, mainly because Bayesian admixture analyses are powerful to assess levels of population differentiation, even when reference parental groups cannot be sampled. At the same time, results are not influenced by the proportion of hybrids in the sample (Pritchard *et al.* 2000; Anderson & Thompson 2002; Corander & Martinnen 2006; Vähä & Primmer 2006). This is certainly important in wildcat hybridization studies because, on the one hand, it is possible to sample domestic cats of 'pure' origin but not to genotype baseline samples of pure wildcats (Beaumont *et al.* 2001) and, on the other hand, there is a high variability in admixture rates. This reflects the need to use advanced methods not sensitive to those variables.

According to the Iberian Red Books of Vertebrates, the European wildcat is considered vulnerable in Portugal (Cabral *et al.* 2005) and near threat in Spain (Palomo & Gisbert 2005). These classifications are mainly based on the low density and fragmentation of populations and the consequent high risk of extinction through hybridization with the copious and pervasive domestic form (Cabral *et al.* 2005; Palomo & Gisbert 2005). The conservation status of the species reflects the importance and urgency to understand the structure and dynamics of the Iberian populations; nevertheless, many ecological and genetic features are still poorly known. Admixture analyses performed in our first study of Portuguese wildcats revealed that hybridization is not frequent and widespread, at least in most recent generations. However, four cryptic hybrids were identified in different geographical areas and an evident sympatry between wildcats and its domestic counterpart was detected (Oliveira *et al.* 2007).

Starting from reference molecular data in Oliveira *et al.* (2007), we extended the admixture analyses to other areas in the Iberian Peninsula by improving both sample size and the geographical range. In this study, we present a first descriptive step to aid the regional and global conservation of this small feline in the Iberian Peninsula by investigating, for the first time, the differentiation between wild and domestic cats and by further evaluating the degree and extent of introgressive hybridization across different areas in Iberia. Additionally, we infer the power and limits of Bayesian admixture analyses to successfully identify admixed genotypes in our dataset, and discuss the usefulness of this study as a model to continue the development of DNA-based tools to detect and monitor hybridization.

2. MATERIAL AND METHODS

(a) *Sampling and individual multilocus genotyping*

We analysed a total of 184 tissue, blood and swab samples from domestic and putative wildcats, which were collected in the Iberian Peninsula in the period of 1993–2006. Sampling comprised 44 wildcats from North (4), Centre (9) and South (31) of Portugal; 31 wildcats from Granada (22), Asturias (3) and Basque Country (6); and 109 feral and pure-bred domestic cats distributed across the Iberian Peninsula (figure 1). This new sample set corresponds to an increase of approximately 50% relative to the one analysed in Oliveira *et al.* (2007). Putative wildcat samples were opportunistically collected or were obtained from collaborative ecological studies. Since wildcats live in low densities and fragmented populations in Iberia, and considering that they are elusive and rare animals, obtaining a larger sample size from this feline is a difficult task and implies long, extensive and persistent efforts. The samples were morphologically identified by collectors according to their wild coat phenotype (Ragni & Possenti 1996), biometrics and geographical location, independently from any genetic information. Four of them were indicated as possible hybrids: FSI711; FSI719; FSI725; and FSI878. Domestic cat samples were obtained from cat pounds, private owners and road-killed animals, including individuals living in sympatry with wildcats (collected in small isolated rural villages).

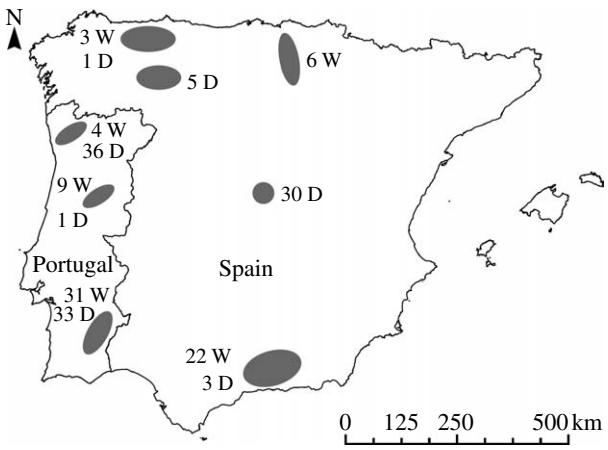

Figure 1. Sampling locations of the studied cats in the Iberian Peninsula (W, wildcats; D, domestic cats).

Total genomic DNA was extracted using a standard salting-out protocol (Sambrook *et al.* 1989) or guanidine thiocyanate (Gerloff *et al.* 1995). A battery of 12 autosomal unlinked microsatellites, formerly isolated and characterized in the domestic cat (Menotti-Raymond & O'Brien 1995; Menotti-Raymond *et al.* 1999), was used to obtain individual multilocus genotypes. Polymerase chain reaction amplifications for each locus were performed following Randi *et al.* (2001). Fragments were separated by size on an ABI 3130*xl* sequencer and genotypes were analysed using GENEMAPPER v. 4.0 (Applied Biosystems).

(b) *Analysis of genetic partition and multivariate clustering*

Allele frequencies, allelic richness, standard diversity indices and expected heterozygosities (He) for each locus were calculated using GenAlEx 6b4 (Peakall & Smouse 2006). Guo & Thompson's (1992) exact test was implemented in GENEPOP v. 3.4 (Raymond & Rousset 1995) in order to statistically evaluate deviations from Hardy–Weinberg equilibrium for all locus–population combinations and to infer pairwise linkage disequilibrium for all loci. Significance levels were adjusted using the sequential method of Bonferroni for multiple comparisons in the same dataset (Rice 1989). GenAlEx 6b4 and GENEPOP v. 3.4 were used to compute F_{ST} (Weir & Cockerham 1984) between wild and domestic

cat populations. Partition of microsatellite diversity between and within wild and domestic populations was estimated through an analysis of molecular variance (AMOVA) on Euclidean pairwise genetic distances, using Φ analogues of Wright's *F*-statistics. Significance testing was done by random permutation. Principal component analysis (PCA) applied to individual multilocus genotypes was also computed in GenAlEx 6b4 in order to describe genetic variation among populations. Wildcats were grouped into two different ways for substructure analysis: (i) wildcats from Portugal (Fsi PT) versus wildcats from Spain (Fsi SP) and (ii) wildcats from Northern Iberia (North and Centre of Portugal, Asturias, León and Basque Country) versus wildcats from Southern Iberia (South of Portugal and Granada).

(c) *Population structure and Bayesian admixture analyses*

Bayesian-based analyses of population structure and admixture proportions were performed using STRUCTURE v. 2.1. (Pritchard *et al.* 2000; Falush *et al.* 2003). All analyses were computed using the admixture model and assuming that allele frequencies are correlated among populations. Using software settings previously described in Oliveira *et al.* (2007), the number of discrete genetic clusters (K) present in the total sample was estimated, with $K=1$–8. The probability of the data for the diverse values of K was analysed using the formula $\mathrm{LnP}(D)_K - \mathrm{LnP}(D)_{K-1}$ (which represents the difference between the likelihood of the data for two consecutive numbers of genetic clusters; Garnier *et al.* 2004). Likelihood values for all inferred K were also converted into probabilities (Pritchard & Wen 2003). Then, for the selected K values, we estimated the membership proportion (Q) of the sampled populations into the detected clusters, and the individual membership proportion q (the proportion of each individual genome that has ancestry in those clusters) was used as a metric of cats sorting into each genetic group. Following simulation results (see below) and previous studies (Pierpaoli *et al.* 2003; Lecis *et al.* 2006; Oliveira *et al.* 2007), an inclusive threshold of $q>0.80$ (and its 90% confidence interval (CI)) was used to assign each individual genotype to one single cluster. Admixed genotypes were detected when an individual proportion of membership was partitioned and lower than 80% to each genetic group (for details on computation and model interpretation, see Oliveira *et al.* 2007).

The inherent drawback of the Bayesian approach is that the validity of the assumed priors and the efficiency of analysed loci cannot be statistically assessed; consequently, simulations have to be implemented for each empirical dataset in order to evaluate the statistical limit of that particular study (Nielsen *et al.* 2006). We assessed the power of the markers and models used in the admixture analyses to distinguish among parental and hybrid classes, and we established the range of q-values expected for all possible admixture generations by simulating both parental and hybrid genotypes in HYBRIDLAB v. 1.0 (Nielsen *et al.* 2006). Based on individual multilocus genotypes, the program initially estimates, locus by locus, allele frequencies for each of the parental wild and domestic populations. Afterwards, multilocus F_1 hybrid genotypes are created by randomly selecting one allele from each of the two populations, according to their frequency distribution (Nielsen *et al.* 2006). Simulations of other hybrid classes (F_2 and backcrosses genotypes) can be computed by the successive use of simulated genotypes as starting-point populations. Briefly, we selected 40 parental domestic and 40 parental wildcats to generate 100 genotypes of each hybrid class: F_1; F_2; and backcrosses. True parental genotypes were selected among cats that revealed individual membership proportions (and their 90% CI) higher than 90% in STRUCTURE, in order to avoid biases caused by possible undetected hybrids. With $K=2$, simulated genotypes were then used in STRUCTURE without any prior non-genetic information, aiming to assess the efficiency of the admixture analyses to estimate the proportion of hybrids in the simulated dataset (see Barilani *et al.* (2007) for further details).

3. RESULTS

In a first exploratory Bayesian analysis, three phenotypically preclassified wildcats (FSI728, FSI729 and FSI737) were significantly assigned to the domestic cluster (individuals' $q_w>0.95$; $p<0.80$). All these samples were collected in Granada province in areas of wildcat distribution. Based on previous results and the considerable error documented for unequivocal phenotypic distinction between European wild and tabby domestic cats (Ragni 1993; Lecis *et al.* 2006), wrong morphological identification was considered the most conservative interpretation and plausible explanation for this incongruence (see §4). Consequently, these three individuals were excluded from the analysis and the total dataset became constituted of 181 samples: 109 domestic cats and 72 wildcats.

(a) *Genetic diversity and multivariate clustering of individual genotypes*

All diversity estimations and differentiation values were corrected after excluding the individuals whose hybrid ancestry could be detected in the Bayesian analysis presented in §3*b*. All loci were polymorphic in both European wild and domestic cats, showing a mean of 5.25 alleles per locus. None of the combinations between pairs of loci disclosed a significant deviation from linkage equilibrium. High levels of expected heterozygosity were found among Portuguese wild, Spanish wild and domestic cats ($He=0.759\pm0.025$, 0.707 ± 0.035 and 0.771 ± 0.028, respectively). Although most of the variation was found within populations (80%), results reflect distinct gene pools among the sampled groups. Over all loci, a highly significant proportion of the total genetic variation was partitioned between wild and domestic populations ($F_{ST(\text{Fca versus Fsi})}=0.20$; $p<0.001$). Multilocus pairwise interpopulation differences were also significant between Portuguese and Spanish wildcats and all domestic cats: $F_{ST(\text{Fca versus Fsi PT})}=0.20$; $F_{ST(\text{Fca versus Fsi SP})}=0.24$; $F_{ST(\text{Fsi PT versus Fsi SP})}=0.11$ ($p<0.001$; figure 2). Significant genetic differentiation was also found when grouping wildcats from Northern (North and Centre of Portugal, Asturias, León and Basque Country) and Southern Iberia in two separated populations ($F_{ST}=0.10$; $p<0.001$; AMOVA). However, genetic closeness between samples was independent of their geographical proximity and genotypes division was more random than the one observed when grouping Portuguese versus Spanish cats (data not shown). Simultaneously, the partition of wildcats into two different clusters obtained in the Bayesian analyses roughly corresponds to the separation of wildcats from Portugal and Spain (see §3*b*). Accordingly, we decided

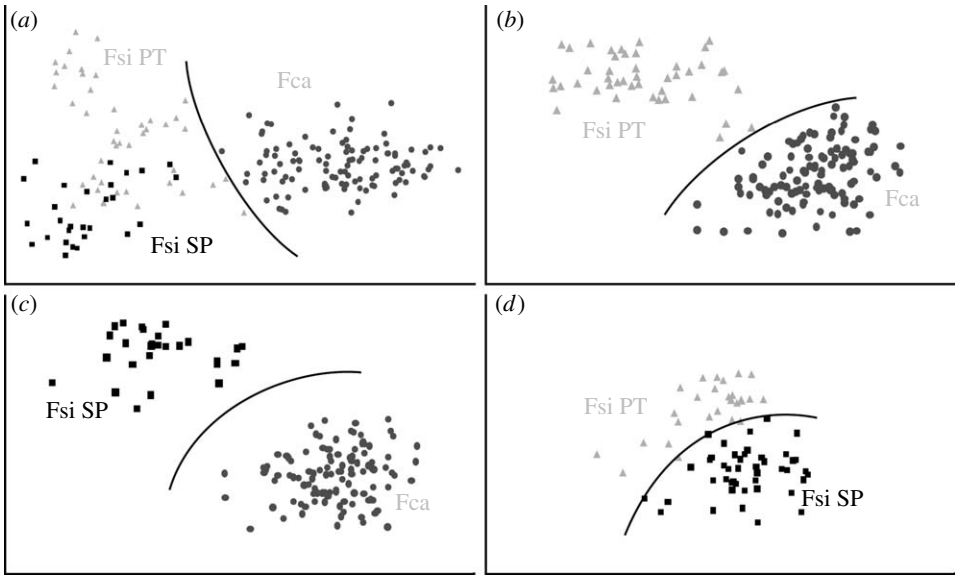

Figure 2. PCA of individual multilocus genotypes, computed using GenAlEx 6b4 (Peakall & Smouse 2006), and genetic divergence (F_{ST}) among the different wild and domestic populations, assessed by AMOVA (Fca, domestic cats; Fsi PT, wildcats from Portugal; Fsi SP, wildcats from Spain): (*a*) plot of Fca and Fsi (from Portugal and Spain; $F_{ST}=0.20$; AMOVA); (*b*) plot of Fca and Fsi PT, approximate area (shaded oval) of expected admixed genotypes and possible hybrid cats ($F_{ST}=0.20$; AMOVA); (*c*) plot of Fca and Fsi SP ($F_{ST}=0.24$; AMOVA); (*d*) plot of Fsi PT and Fsi SP ($F_{ST}=0.11$; AMOVA).

to nominate the samples as Portuguese and Spanish cats. PCA scores of all individuals were graphically presented in a dimensional plot defined by two principal axes that explain, cumulatively, 55.58% of the total genetic variability (figure 2*a*). The plotting disclosed an evident separation between wild (Fsi SP+Fsi PT) and domestic cat populations, revealing a clear genetic differentiation between them. A closer proximity was found between Portuguese wildcats and domestic cats, when compared with the genetic proximity between Spanish wild and domestic cats (figure 2*b*,*c*). Some putative Portuguese wildcats plotted towards the domestic group, corresponding to outlier individuals that might have admixed ancestry (figure 2*b*; see Bayesian analysis in §3*b* for outlier individuals' identification).

(b) *Bayesian inference of population structure and admixture*

Bayesian admixture analyses using only genetic information clearly suggested the presence of two or three sharply differentiated groups in the Iberian Peninsula, since the probability of the data increased steadily for $K=2$ and 3. Afterwards, the difference between likelihood values of consecutive values of K reached a plateau. At the same time, the conversion of likelihood values into probabilities, following Pritchard & Wen (2003), revealed very high probabilities ($p>99.9\%$) of having two or three distinct clusters in the dataset, against almost 0.00% for higher values of K. With $K=2$, and using only genetic information, we estimated the average membership proportions (Q) of each predefined group (wild and domestic cats) into both clusters genetically inferred. All domestic cats were probabilistically assigned to cluster I, with $Q_I=0.99$, while wildcats were mostly assigned to cluster II, with $Q_{II}=0.96$. Therefore, splitting the samples into two clusters allowed assigning individuals to their biological

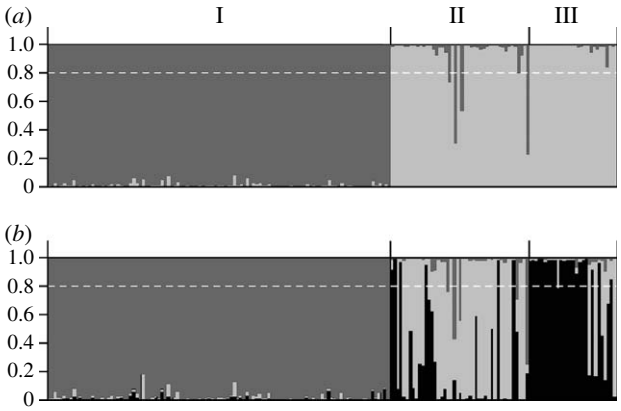

Figure 3. Probabilistic assignment of wild and domestic cats to the genetic clusters inferred by the Bayesian analysis performed in STRUCTURE, with (*a*) $K=2$ and (*b*) $K=3$. Each cat is represented by a vertical bar fragmented in K coloured sections that are relative to their membership proportion in the diverse genetic clusters: I, domestic cluster (dark grey); II, Portuguese wildcat (light grey); III, Spanish wildcat (black). The horizontal white line represents the threshold probability of 80% used to assign each individual to a single population.

partition as wild and domestic cats. For $K=3$, all domestic cats were equally assigned to cluster I while wildcat samples were further subdivided between clusters II and III, with Portuguese cats clustering with $Q_{II}=0.683$ and $Q_{III}=0.250$ and Spanish wildcats assigning with $Q_{II}=0.169$ and $Q_{III}=0.817$ (figure 3*a*,*b*). Partition assignment of some Spanish genotypes in cluster II and Portuguese cats in cluster III does not reflect a closer geographical origin to the other nominal population.

At a probabilistic threshold of $q_i>0.80$, the admixture analysis performed on simulated genotypes was able to efficiently recognize 100% of the parental individuals, and their 90% CI were higher than 0.88. All the F_1 hybrids were correctly identified as admixed

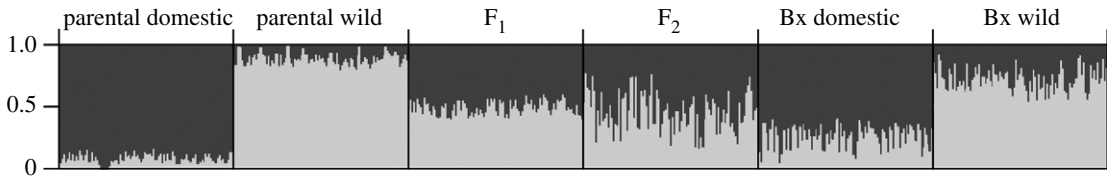

Figure 4. Plot of the Bayesian analyses performed in STRUCTURE using simulated parental, F_1, F_2 and backcrosses (Bx) genotypes. The program was computed for $K=2$ under the admixture model. Each individual is represented by a vertical bar coloured according to the proportion of its genome descending from K clusters. Genotypes were simulated using HYBRIDLAB v. 1.0 (Nielsen *et al.* 2006).

Table 1. Individual assignment and inferred ancestry of sampled wild and domestic cats, indicating the individuals identified as admixed cats. (Individual q_i values were calculated in STRUCTURE, supplying or excluding prior population information (ancestry in the other population: migrant, sampled generation; F_1, first-generation hybrid; F_2, second-generation hybrid). Cluster I, domestic cats; cluster II, wildcats (values in brackets represent 90% CIs).)

modelling	samples	cluster I	cluster II
without prior non-genetic information	domestic cats ($n=109$)	0.987 (0.890–1.000)	0.013 (0.000–0.210)
	wildcats ($n=67$)	0.041 (0.000–0.130)	0.959 (0.870–1.000)
	FSI682	0.695 (0.587–0.760)	0.305 (0.240–0.413)
	FSI685	0.448 (0.345–0.701)	0.552 (0.299–0.655)
	FSI689	0.547 (0.270–0.698)	0.453 (0.302–0.730)
	FSI711	0.696 (0.384–0.755)	0.304 (0.245–0.616)
	FSI878	0.709 (0.597–0.759)	0.291 (0.241–0.403)

			other population		
with information for:		population of origin	Migrant	F_1	F_2
all individuals	domestic cats ($n=109$)	0.978	0.000	0.001	0.021
	wildcats ($n=67$)	0.981	0.000	0.004	0.015
	FSI682	0.048	0.100	0.317	0.320
	FSI685	0.362	0.001	0.766	0.174
	FSI689	0.191	0.138	0.265	0.405
	FSI711	0.160	0.034	0.503	0.304
	FSI878	0.025	0.091	0.112	0.772
all except hybrids	domestic cats ($n=109$)	0.979	0.000	0.001	0.020
	wildcats ($n=67$)	0.972	0.000	0.007	0.021
	FSI682	0.576 (0.383–0.765)	0.424 (0.235–0.617)		
	FSI685	0.503 (0.313–0.691)	0.497 (0.309–0.687)		
	FSI689	0.502 (0.313–0.697)	0.498 (0.303–0.687)		
	FSI711	0.536 (0.344–0.729)	0.464 (0.271–0.656)		
	FSI878	0.771 (0.245–0.799)	0.229 (0.755–0.211)		

cats; however, a proportion of 9% F_2 and 15% backcross genotypes showed a $q_i > 0.80$ to one single cluster and could not be distinguished from their parentals (figure 4). All hybrids detected by simulations revealed a very wide 90% CI, ranging between 0.20 and 0.80. Accordingly, we performed admixture analyses using the threshold of $q_i > 0.80$.

Values of individual proportion of membership q_i and their 90% CI computed with $K=2$–3 showed that all domestic cats had $q_d > 0.80$ and a minimum 90% CI of 0.89. Cluster II grouped approximately 93% of the phenotypically classified wildcats (90% CI between 0.87 and 1.00). Among Portuguese wildcats, we found five exceptions to this sharp differentiation, since putative wildcats FSI682, FSI685, FSI689, FSI711 and FSI878 revealed a wild assignment and 90% CI < 0.80, disclosing significant values for domestic ancestry (table 1; figure 3*a*). At the same time, while most of the 90% CI values ranged between 0.87 and 1.00, putatively admixed cats revealed wider credibility intervals, ranging from 0.27 to 0.73. Individuals FSI682, FSI685, FSI689 and FSI878 had been identified as outliers in the PCA plotting (figure 2*b*). In a more stringent modelling approach, using prior morphological identification for all sampled wildcats (USEPOPINFO = 1) and including or excluding the information for the putative hybrids formerly identified (POPFLAG = 0 or 1), all posterior probabilities confirmed the results based only on genetic classification and the five admixed cats also revealed a q_w and 90% CI < 0.80 (table 1). Even though the ancestral class of hybrid genotypes can be assessed, either in current or first and second past generations, none of these individuals presented a posterior probability above 0.80 for a single past hybrid generation. In any case, putative wildcat FSI682 presented a considerable

superior probability of being an F_1 hybrid ($q_{F1}=0.77$) and FSI878 of being a backcross with the domestic gene pool ($q_{Bxd}=0.78$). Assuming that probabilistic assignments below the threshold indicate admixture, a minimum of 2.8% of the Iberian cats sampled in this study showed signals of introgressive hybridization.

4. DISCUSSION

(a) *Hybridization in wildcats from Iberian Peninsula*

The preservation of wildlife populations has always been a controversial issue, mainly because many factors must be taken into consideration to design efficient management programmes. Nevertheless, it is a general agreement that conservation measures should focus on preserving healthy and outbred populations essentially by maintaining sufficiently large and suitable habitats that allow genetic exchange. The protection of some wildlife environments not only benefits that particular species, but also assists the preservation of important ecosystems and other cohabitant species. Unfortunately, the European continent has undergone significant habitat loss and fragmentation over the years, impeding the natural range of most wildlife species. Thus, a variety of wildcat populations has an extremely limited natural range and lives in low densities.

Along with habitat preservation, the maintenance of genetically unique and pure wild populations is recognized as a high conservation priority. Artificial hybridization between a species and its domesticated equivalent can severely influence the conservation status of threatened species and their legal protection. The introgression of alien domestic alleles has even led to extinction of many populations and species (see Rhymer & Simberloff 1996; Allendorf *et al.* 2001). The risk of introgression of domestic cat genes into wildcat gene pools is a big concern to conservation biologists, since most wildcat populations are now in juxtaposition with the urban ranges of feral domestic cats (Stahl & Artois 1994). Cross-breeding with domesticated forms may culminate in the homogenization of gene pools and result in outbreeding depression, reduced fitness and, consequently, severe population declines of wild populations (Barilani *et al.* 2007). Especially for closely related (sub)species, identifying the ecological and biological driving forces of this phenomenon can be exceptionally challenging. Being able to understand these factors or even to identify such admixture events is particularly complex in domestic and wildcat subspecies, considering their significant genetic proximity when compared with other hybridizing *taxa* (e.g. grey wolf (*Canis lupus*) and dog (*Canis familiaris*): Verardi *et al.* 2006; coyote (*Canis latrans*) and red wolf (*Canis rufus*): Adams *et al.* 2007).

In order to develop population management programmes for European wildcats, the uniqueness and 'genetic purity' of populations needs to be evaluated. Here, we successfully performed Bayesian admixture analyses of empirical and simulated datasets using microsatellite multilocus genotypes from wild and domestic cats across the Iberian Peninsula. Our findings confirm the conclusions documented in previous hybridization studies, showing that, for populations with F_{ST} values approximately 0.12–0.20, 12–24 loci are sufficient to detect first-generation hybrids (Vähä & Primmer 2006; Barilani *et al.* 2007; Oliveira *et al.* 2007). However, the detectability of hybrids decreases exponentially with repeated backcrossing into the parental groups, and beyond the second generation of hybridization some individuals classified as pure wildcats might actually result from repeated backcrosses of admixed cats with wild individuals. Even though we were able to improve our first analyses by increasing the representatives of both parental populations (see Oliveira *et al.* 2007), only a slight increase in F_2 (88–91%) and backcrosses (80–85%) identification was achieved in the simulation tests, and it was not enough to unambiguously identify all hybrid classes. Accordingly, it is essential to find the means to continue increasing the power of Bayesian analyses to differentiate and detect admixture between wild and domestic cats. For genotypes with no missing data, larger CIs are expected in admixed individuals, mainly if the parental populations are not sampled (Pritchard *et al.* 2000; Barilani *et al.* 2007). The wide 90% CI observed for all simulated hybrids confirms these findings. The correct estimation of membership of all the unequivocally preclassified domestic cats also corroborates the efficiency of the analyses.

Hybridization can be overall widespread or locally very rare, which can be related to the specific circumstance in which cross-breeding occurs. Mapping levels of introgression across European wildcat populations can be used to prioritize areas for preservation and perform focused and efficient conservation strategies. Empirical population structure analyses showed that Iberian wild and domestic cats have high average posterior probabilities of assignment to their parental clusters, belonging to two clearly separated gene pools. Thus, we may assume that genetically distinct European wildcats remain in the Iberian Peninsula and populations are scarcely hybridized in the most recent generations. Nevertheless, using both a stringent procedure where prior population information is given and without using any non-genetic information, at least 6.9% (5 out of 72; 2.8% of all samples) of the Iberian wildcats probably have hybrid ancestry. These findings add an admixed cat to the four already detected in the Portuguese wildcat population by Oliveira *et al.* (2007), an individual that was sampled in a natural park in the Centre of Portugal. These admixed individuals probably represent diverse levels of hybridization, suggesting that cross-breeding exists and should be regarded as a real threat to the wild population. Hybrid cats were exclusively identified in Portugal and closer genetic similarity was found between Portuguese wild and domestic cats, which might be an indication of higher levels of recent introgression when compared with Spain. Small localized populations are known to be more susceptible to decline through hybridization and Portuguese populations are thought to be decreasing, increasingly fragmented and isolated. According to our simulations on F_2 and backcrosses detection, we regard this number of hybrids as a minimum value of admixed cats in Portugal, since past events of cross-breeding might have remained undetected if hybrids are

backcrossing with individuals that belong to the parental populations. Geographically separated wildcat populations from Portugal and Spain revealed a genetic divergence that suggests they should be considered singular units of study. However, some of the Portuguese and Spanish cats were assigned to the other cluster without any apparent biological/ecological reason, such as translocation or geographical proximity of animals. In our perspective, this can be explained by two simultaneous reasons. On the one hand, we are dealing with populations that are genetically very close and, although significant divergence was found (significant F_{ST} values in AMOVA and $K=3$ in STRUCTURE), splitting of genotypes is still not absolute and not sufficient to divide wildcats in completely non-overlapping separated groups. On the other hand, and as a consequence of the mentioned genetic similarity, the number and type of molecular markers need to be increased to be able to perform fine substructure analysis across wildcat populations.

According to the genetic data, three of the morphologically preclassified Spanish wildcats were significantly assigned to the domestic cluster. Only two of the genetically admixed cats were morphologically identified as possible hybrids (FSI711 and FSI878) and two morphological hybrids were genetically classified as wildcats with very high membership probabilities (FSI719 and FSI725; $q_w > 0.96$, minimum 90% CI of 0.93). In a wildcat population with admixture, there is the possibility that the putative wildcats that are genetically assigned to the domestic cluster represent, in fact, backcrosses, which we were not able to detect due to the discussed limitations in the discriminatory power of our analyses. However, no hybrids of any class (F_1, F_2 or backcrosses) were identified in most recent generations among the Spanish cats. Particularly from the Granada population, where these three cats were collected, we have genotyped 19 more individuals and no evidences of recent admixture were found. Accordingly, although we cannot totally reject the hypothesis of past undetected admixture, we think that the most conservative way of dealing with these results is to exclude these cats from the analyses, since they probably represent wrong morphological identifications other than wrong molecular assignments. These discrepancies have been previously referred to in wildcat studies (Ragni 1993; Lecis *et al.* 2006) and highlight the importance of using molecular tools for wild, domestic cats and cryptic hybrids identification.

(b) *Ongoing development of wildcat molecular studies*

Many demographic, ecological and historical reasons might be involved in the diverse hybridization and introgression rates found across European wildcat populations: (i) it is possible that habitat changes and fragmentation may have had higher impact on original forest landscapes (Central Europe) than on mosaic Mediterranean landscapes (Southern Europe), (ii) the tradition to have house cats or to feed feral domestic cats is different in different places and can also be an important variable, (iii) the different demographic declines that European wildcat populations underwent may have allowed feral domestic cats to cross-breed differently in the past, when populations comprised just a few reproductive individuals, and (iv) the multi-domestication events recently described by Driscoll *et al.* (2007) might have resulted in diverse domestic cat gene pools that may have introgressed differently into the wild populations across Europe.

Different methodological developments may contribute to a substantial improvement of the population analysis presented in this study and to address several questions regarding hybridization rates across European wildcat populations. A two-pronged molecular approach (using both invasive and non-invasive sampling procedures) would be of major importance to monitor populations of this endangered and elusive feline. Scat surveys allow a time and cost-effective sampling effort in inconspicuous populations and they significantly reduce anthropogenic pressures related to wildlife trapping and handling. At a molecular level, it is crucial to overcome identification uncertainties searching for more powerful diagnostic traits. The ability to identify hybridization further back in the past using neutral unlinked microsatellites would imply a significant higher genotyping effort, as suggested by Rosenberg *et al.* (2003) and more recently by Vähä & Primmer (2006). Accordingly, a simultaneous increase in the number and type of analysed loci would be necessary to discriminate between hybrid classes and to develop high-resolution inferences, especially if combined with recently available statistical methods based on Bayesian assumptions (Pritchard *et al.* 2000; Vähä & Primmer 2006). The use of a large number of unlinked and linked microsatellites may allow better estimates of individual cats' assignments and genotyping microsatellites located in linkage groups might enable better statistical estimates of hybridization further back in the past (Lecis *et al.* 2006). At the same time, a genome-wide investigation of novel molecular markers and the establishment of new diagnostic loci are our current field of investigation. Domestication produced obvious changes in the reproduction, coat colour, size, disease resistance and behaviour of domestic cats, when compared with the ancestral wildcat. Therefore, we will perform a detailed analysis of polymorphism at candidate genes underlying several domestic traits, determine current patterns of diversity in such genes and search for genetic footprints in cat's genome, i.e. signatures of selection that may have happened during domestication events at these loci. This analysis will focus on candidate genes identified as having major functional roles in mammal species, namely the ones most probably involved in litter sizes, fertility and coat colour patterns diversity. Following this line of research, we aim to identify single nucleotide polymorphisms, molecular markers that might overcome some technical errors inherent to microsatellites (e.g. size homoplasy) and that have been revealing high efficiency, genotyping facility and analytical simplicity in their gradual application in population structure and admixture analyses (Zhang & Hewitt 2003). Furthermore, the usefulness of all newly identified markers can be used to develop a simple and rapid protocol (based on the most informative loci) as

a routine DNA-based test to detect and monitor hybridization in the wild.

It is also important to point out that the consolidation of molecular inferences should include an extensive ecological knowledge of wildcat populations. More focused conservation policies might be achieved through the identification of historical and recent ecological features that could be related to admixture events and promote them. A more extensive study should aim to relate habitat variables to hybridization, by using a comparative analysis of scarcely admixed versus largely hybridized populations in both less modified and disturbed landscapes across the entire Iberian Peninsula.

All wildcat samples were collected from road-killed animals or provided by the Portuguese National Tissues Bank, under the ethical rules from Portugal and Spain. Domestic cat samples were collected either from road-killed animals or using precise veterinarian practices.

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REFERENCES

Adams, J. R., Lucash, C., Achutte, L. & Waits, L. P. 2007 Location hybrid individuals in the red wolf (*Canis rufus*) experimental population area using spatially targeted sampling strategy and faecal DNA genotyping. *Mol. Ecol.* **16**, 1823–1834. (doi:10.1111/j.1365-294X.2007.03270.x)

Allendorf, F. W., Leary, R. F., Spruell, P. & Wenburg, J. K. 2001 The problems with hybrids: setting conservation guidelines. *Trends Ecol. Evol.* **16**, 613–622. (doi:10.1016/S0169-5347(01)02290-X)

Anderson, E. C. & Thompson, E. A. 2002 A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160**, 1217–1229.

Barilani, M., Sfougaris, A., Giannakopoulos, A., Mucci, N., Tabarroni, C. & Randi, E. 2007 Detecting introgressive hybridisation in rock partridge populations (*Alectoris graeca*) in Greece through Bayesian admixture analyses of multilocus genotypes. *Conserv. Genet.* **8**, 343–354. (doi:10.1007/s10592-006-9174-1)

Beaumont, M., Barratt, E. M., Gottelli, D., Kitchener, A. C., Daniels, M. J., Pritchard, J. K. & Bruford, M. W. 2001 Genetic diversity and introgression in the Scottish wildcat. *Mol. Ecol.* **10**, 319–336. (doi:10.1046/j.1365-294x.2001.01196.x)

Cabral, M. J. *et al.* (eds) 2005 *Livro Vermelho dos Vertebrados de Portugal*. Lisboa, Portugal: Instituto de Conservação da Natureza e Biodiversidade.

Corander, J. & Martinnen, P. 2006 Bayesian identification of admixture events using multilocus molecular markers. *Mol. Ecol.* **15**, 2833–2843. (doi:10.1111/j.1365-294X.2006.02994.x)

Daniels, M. J., Balharry, D., Hirst, D., Kitchener, A. C. & Aspinall, R. J. 1998 Morphological and pelage characteristics of wild living cats in Scotland: implications for defining the 'wildcat'. *J. Zool.* **244**, 231–247. (doi:10.1017/S0952836998002088)

Daniels, M. J., Beaumont, M. A., Johnson, P. J., Balharry, D., Macdonald, D. W. & Barratt, E. 2001 Ecology and genetics of wild-living cats in the north-east of Scotland and the implications for the conservation of the wildcat. *J. Appl. Ecol.* **38**, 146–161. (doi:10.1046/j.1365-2664.2001.00580.x)

Driscoll, C. A. *et al.* 2007 The Near Eastern origin of cat domestication. *Science* **317**, 519–523. (doi:10.1126/science.1139518)

Falush, D., Stephens, M. & Pritchard, J. 2003 Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**, 1567–1587.

Garnier, S., Alibert, P., Audiot, P., Prieur, B. & Rasplus, J. Y. 2004 Isolation by distance and sharp discontinuities in gene frequencies: implications for the phylogeography of an alpine insect species, *Carabus solieri*. *Mol. Ecol.* **13**, 1883–1897. (doi:10.1111/j.1365-294X.2004.02212.x)

Gerloff, U., Schlötterer, C., Rassmann, K., Rambold, I., Hohmann, G., Frutth, B. & Tautz, D. 1995 Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living Bonobos (*Pan paniscus*). *Mol. Ecol.* **4**, 515–518. (doi:10.1111/j.1365-294X.1995.tb00247.x)

Guo, S. W. & Thompson, E. A. 1992 Performing the exact test of Hardy-Weinberg proportions for multiple alleles. *Biometrics* **48**, 361–372. (doi:10.2307/2532296)

Lecis, R., Pierpaoli, M., Birò, S., Szemethy, L., Ragni, B., Vercillo, F. & Randi, E. 2006 Bayesian analyses of admixture in wild and domestic cats (*Felis silvestris*) using linked microsatellite loci. *Mol. Ecol.* **15**, 119–131. (doi:10.1111/j.1365-294X.2005.02812.x)

McOrist, S. & Kitchener, A. C. 1994 Current threats to the European wildcat in Scotland. *Biol. Conserv.* **76**, 212.

Menotti-Raymond, M. A. & O'Brien, S. 1995 Evolutionary conservation of ten microsatellites loci in four species of Felidae. *J. Hered.* **86**, 319–322.

Menotti-Raymond, M., David, V. A., Lyons, L. A., Schäffer, A. A., Tomlin, J. F. & O'Brien, S. J. 1999 A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). *Genomics* **57**, 9–23. (doi:10.1006/geno.1999.5743)

Nielsen, E. E., Bach, L. A. & Kotlicki, P. 2006 HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. *Mol. Ecol. Notes* **6**, 971–973. (doi:10.1111/j.1471-8286.2006.01433.x)

Oliveira, R., Godinho, R., Randi, E., Ferrand, N. & Alves, P. C. 2007 Molecular analysis of hybridisation between wild and domestic cats (*Felis silvestris*) in Portugal: implications for conservation. *Conserv. Genet.* **9**, 1–11. (doi:10.1007/s10592-007-9297-z)

Palomo, L. J. & Gisbert, J. (eds) 2005 *Atlas de los Mamíferos Terrestres de España*. Madrid, Spain: Dirección General de Conservación de la Naturaleza- SECEM-SECEMU.

Peakall, R. & Smouse, P. E. 2006 GenAlEx 6: genetic analysis in EXCEL. Population genetic software for teaching and research. *Mol. Ecol. Notes* **6**, 288–295. (doi:10.1111/j.1471-8286.2005.01155.x)

Pierpaoli, M., Birò, Z. S., Herrmann, M., Hupe, K., Fernandes, M., Ragni, B., Szemethy, L. & Randi, E. 2003 Genetic distinction of wildcat (*Felis silvestris*) populations in Europe, and hybridization with domestic cats in Hungary. *Mol. Ecol.* **12**, 2585–2598. (doi:10.1046/j.1365-294X.2003.01939.x)

Pritchard, J. & Wen, W. 2003 *Documentation for STRUCTURE software*, version 2. Chicago, IL: Department of Human Genetics, University of Chicago.

Pritchard, J., Stephens, M. & Donnelly, P. 2000 Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.

Ragni, B. 1993 The crucial problem of *in vivo* identification of wildcat and recognition of hybrids with domestic cats. Peruglia, Italy: Institute of Zoology, Peruglia University.

Ragni, B. & Possenti, M. 1996 Variability of coat-colour and markings system in *Felis silvestris*. *Ital. J. Zool.* **63**, 285–292.

Randi, E., Pierpaoli, M., Beaumont, M. & Ragni, B. 2001 Genetic identification of wild and domestic cats (*Felis silvestris*) and their hybrids using Bayesian clustering methods. *Mol. Biol. Evol.* **18**, 1679–1693.

Raymond, M. & Rousset, F. 1995 Genepop (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**, 248–249.

Rhymer, J. M. & Simberloff, D. 1996 Extinction by hybridisation and introgression. *Annu. Rev. Ecol. Syst.* **27**, 29–44. (doi:10.1146/annurev.ecolsys.27.1.83)

Rice, W. R. 1989 Analyzing tables of statistical tests. *Evolution* **43**, 223–225. (doi:10.2307/2409177)

Rosenberg, N. A., Li, L. M., Ward, R. & Pritchard, J. K. 2003 Informativeness of genetic markers for inference of ancestry. *Am. J. Hum. Genet.* **73**, 1402–1422. (doi:10.1086/380416)

Sambrook, E., Fritsch, F. & Maniatis, T. 1989 *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbour, NY: Cold Spring Harbour Press.

Stahl, P. & Artois, M. 1994 Status and conservation of the wildcat (*Felis silvestris*) in Europe and around the Mediterranean rim. Convention on the Conservation of European Wildlife and Natural Habitats Standing Committee. *Council of Europe Nature and Environment Series*, vol. 69, pp. 1–76. Strasbourg, France: Council of Europe Press

Vähä, J. P. K. & Primmer, C. R. 2006 Detecting hybridization between individuals of closely related populations—a simulation study to assess the efficiency of model-based Bayesian methods to detect hybrid individuals. *Mol. Ecol.* **15**, 63–72. (doi:10.1111/j.1365-294X.2005.02773.x)

Verardi, A., Lucchini, V. & Randi, E. 2006 Detecting introgressive hybridisation between free-ranging domestic dogs and wild wolves (*Canis lupus*) by admixture linkage disequilibrium analysis. *Mol. Ecol.* **15**, 2845–2855. (doi:10.1111/j.1365-294X.2006.02995.x)

Weir, B. S. & Cockerham, C. C. 1984 Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370. (doi:10.2307/2408641)

Zhang, D. X. & Hewitt, G. M. 2003 Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Mol. Ecol.* **12**, 563–584. (doi:10.1046/j.1365-294X.2003.01773.x)